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Expression of Tissue Factor, Thrombomodulin, and E-Selectin in Baboons with Lethal *Escherichia coli* Sepsis

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Disseminated intravascular thrombosis is a frequent complication of endotoxic shock, and modulation of endothelial cell hemostatic properties has been proposed to play a role in its pathogenesis based on studies of endothelial cells in culture. This study examined the in vivo expression of tissue factor (TF) and thrombomodulin (TM) in a baboon model of lethal* Escherichia coli *sepsis using immunohistochemistry with monospecific antibodies. Expression of E-selectin (E-sel) was also determined as a marker of endothelial cell activation. Correlation of immunoreactivity with procoagulant activity in lipopolysaccharide-stimulated cultured human endothelial cells showed that immunohistochemistry was sufficiently sensitive to detect as little as 5% of the maximum in vitro endothelial cell TF response. Vascular endothelium of control animals expressed TM but had no detectable TF or E-sel. Following* E. coli *infusion, widespread E-sel expression and microvascular fibrin deposition was evident within 6 hours. However, expression of TF by endothelial cells became detectable only in the splenic microvasculature, where endothelial specificity of TF expression was confirmed by dual immunofluorescence of TF with von Willebrand's factor and with TM. In the spleen, there was a dissociation of expression of TF and E-sel, with marginal zone vessels being TF-positive and E-sel-negative, whereas sinusoidal endothelium was E-sel-positive but TF-negative. TM expression was unchanged from controls. Additionally, expression of TF by lung alveolar epithelial cells, splenic macrophages, and epithelial cells of the renal glomeruli was observed to be enhanced in septic animals. This study documents endothelial cell expression of TF in vivo in a relevant pathological setting. At the same time, compared with endothelial cells in culture, there is in vivo both significantly greater control of TF expression than expected, given the strong positive stimuli present in lethal* E. coli *septic shock and an unpredicted heterogeneity of activation responses. (Am J Pathol 1993, 142:1458–1470)

The syndrome of disseminated intravascular coagulation is a frequent complication of septic shock caused by gram-negative bacteria. In the microvasculature, fibrin thrombi are present adjacent to intact endothelial cells, suggesting that there is dysregulation of endothelial cell anticoagulant function.[1] Two such properties that have been well studied in cultured cells are the expression of tissue factor (TF) and thrombomodulin (TM).[2] Both are integral membrane glycoproteins that are receptors and co-factors for coagulation proteases. TF binds factor VIIa, and the complex initiates the coagulation cascade by activating factors X and IX.[3,4] TM binds thrombin, and the complex activates protein C, leading to the inactivation of factors Va and VIIIa.[5,6] Unstimulated endothelial cells, *in vivo* and in culture, constitutively express TM but lack TF.

Exposure of endothelial cells in culture to endotoxin or tumor necrosis factor (among other agonists) induces the expression of TF and concomitantly decreases the expression of TM.[7,8] These changes oc-

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cur in concert with alterations in the expression of other proteins, including other hemostatic proteins and leukocyte adhesion molecules.[9] Among the latter is E-selectin (E-sel; formerly endothelial leukocyte adhesion molecule-1 or ELAM-1). Like TF, E-sel is an integral membrane protein not expressed by unstimulated endothelium, and the temporal expression of E-sel by lipopolysaccharide- or cytokine-stimulated endothelial cells in culture parallels that of TF.[10] Recognition of these multiple physiological changes, many of which involve the activation or suppression of messenger RNA transcription, has led to the concept of endothelial activation as a basic pathophysiological response of endothelium.[9] Demonstration of E-sel expression in baboons with septic shock supports the hypothesis that endothelial activation occurs in this disease.[11] The current study was undertaken to determine whether endothelium in septic shock also expresses TF and down-regulates TM, whether such changes are linked with E-sel expression, and whether changes were associated with microvascular thrombosis.

Materials and Methods

Animals

Experiments were performed on adolescent baboons (*Papio c. anubis* or *Papio c. cynocephalus*) purchased from a breeding colony maintained by the University of Oklahoma Health Sciences Center Animal Facility. Animals were healthy, having peripheral blood leukocyte counts of 5,000 to 7,000/ml and hematocrits exceeding 36%, and being free of tuberculosis. All were observed for a minimum of ten days after transfer to the Veterans Administration Hospital Animal Facility and were fasted overnight before study.

Septic Shock Protocol

Lethal *Escherichia coli* septic shock was produced as previously described, following protocols approved by the Animal Use Committees of both the Veterans Administration Hospital and the University of Oklahoma Health Sciences Center.[1] Under anesthesia, animals were intubated and had femoral arterial and venous catheters placed. A lethal dose of live *E. coli* was infused intravenously over 2 hours. The initiation of infusion was defined as 0 hours or T-0. Heart rate, blood pressure, temperature, and blood samples were monitored at baseline and hourly until sacrifice. Measured in blood were complete blood counts with leukocyte differentials, platelet counts, fibrinogen, fibrin degradation products, blood urea nitrogen, and quantitative bacterial culture. Animals were sacrificed by lethal infusion of sodium pentobarbital.

Tissue Collection and Processing

In two animals with lethal sepsis, core biopsies (3-mm diameter) of skin, liver, and spleen were obtained before infusion of *E. coli* and at 2 and 6 hours post-infusion via surgical incisions (Table 1, A). Immediately after sacrifice by lethal injection of sodium pentobarbital at T+6 hours, samples of brain, myocardium, lung, adrenal gland, kidney, aorta, and skeletal muscle were taken. These tissues were also obtained from three other experimental animals sacrificed at T+6, T+10, and T+24 hours (Table 1, B), and two sham infected animals at T-0 and T+2 hours (Table 1, C). Sections of tissue measuring approximately 5 × 5 × 2 mm were immersed in OCT compound (Miles, Inc., Elkhart, IN) in cryomolds, frozen in liquid nitrogen, then kept at −70 C.

Table 1. *Characteristics and Tissue Sampling of Animals Studied*

Expt. No.	Date	Wt. (kg)	Sex	*E. coli* (CFU × 10^{10}/kg)	Time of sacrifice (hour)
A. Animals from which serial liver, spleen, and skin biopsies were taken at T-0 and T+2 hours and from all organs at T+6 hours following *E. coli* infusion:					
6	7/89	7.7	M	1.23	6
8	7/90	16.8	F	0.75	6
B. Animals from which tissue samples from all organs were taken at 6, 10, or 24 hours following *E. coli* infusion					
3	3/89	7.3	M	2.76	6
5	5/89	7.7	F	1.37	10
10	4/91	9.3	F	1.32	24
C. Animals (control) from which tissue samples were taken from all organs at 0 or 2 hours following saline infusion:					
4	3/89	7.1	M		0
12	4/91	8.6	F		2

Immunohistochemistry

The primary antibodies utilized were murine monoclonal antibody clones TF9-9C3 and TF9-10H10, derived against human brain TF, and affinity-purified goat anti-human TF, provided by Dr. Thomas Edgington of the Scripps Research Institute, La Jolla, CA, and by Dr. James Morrissey of the Oklahoma Medical Research Foundation[12]; affinity-purified goat anti-rabbit TM, provided by Dr. Naomi Esmon of the Oklahoma Medical Research Foundation, Oklahoma City, OK[13]; murine monoclonal antibodies to human E-sel, clones H4/18 and H18/7, provided by Dr. Michael Bevilacqua of Harvard Medical School[14]; murine monoclonal antibody to fibrin, clone E8, purchased from AMAC, Inc., Westbrook, ME; murine monoclonal antibody to CD68 (a macrophage marker), clone EMB-11, purchased from Dako, Inc., Carpinteria, CA[15]; affinity-purified rabbit anti-human von Willebrand factor, purchased from Dako, Inc.; murine monoclonal antibody to CALLA (CD10), clone SS2/36, purchased from Dako; murine monoclonal antibody to vimentin, clone V9, purchased from Dako; and murine monoclonal antibody to cytokeratin, clone CAM 5.2, purchased from Becton-Dickenson, Mountainview, CA. These were tested initially on similarly processed sections of normal baboon tissue to ensure appropriate recognition in this species. Control antibodies were irrelevant murine monoclonal antibodies of the same class and isotype as the primary monoclonals or purified goat immunoglobulin and were always tested in parallel with specific antibody. Primary specific and control antibodies were used at 10 μg/ml. Characterization and specificity of the monoclonal antibodies to TF and to E-sel have been reported.[12,14] Specificity of the polyclonal antibody to TF was determined by demonstrating identical patterns of reactivity with the monoclonals on baboon and human tissues. Specificity of the goat anti-rabbit TM was tested by documenting lack of immunostaining following in solution absorption with 10-fold excess purified TM. Secondary antibodies were purchased from Vector, Inc., Burlingame, CA, or Dako.

Six- to 10-mm-thick cryosections were fixed in 100% methanol at −20 C for 2 minutes, then air-dried, and used immediately or stored in airtight slide boxes with dessicant at −70 C for not more than 2 weeks. For light microscopy, the Vector ABC Elite system with 3-amino-5-ethylcarbazole as the chromogen was used as previously described.[16] Endogenous peroxidase activity was blocked by incubation of sections with 0.2% hydrogen peroxide in water for 5 minutes following the secondary antibody incubation. For fluorescence microscopy, bound primary antibodies were detected using the appropriate fluorescein- or rhodamine-conjugated secondary antibody, or using an ABC Elite system with fluorescein isothiocyanate- or tetramethylrhodamine isothiocyanate conjugated avidin in the third stage. Secondary antibodies were selected that would not recognize each other or another primary antibody in dual labeling studies. For these studies, sections were incubated first with antibody to TF or E-sel overnight at 4 C, followed sequentially by the biotinylated secondary antibody and the fluorescently labeled avidin complex, then the second primary antibody, and lastly its appropriate secondary antibody, each of the latter 4 incubations being for 1 hour at 37 C, with intervening washes. Controls run with each dual immunofluorescence experiment were set up identically to the test incubations but used irrelevant primary antibodies of the same type as the specific antibodies to ensure there was no nonspecific binding or cross reactivity of the secondary antibodies. In these experiments, nonspecific binding was negligible and no cross reactivity was observed. Leakage of fluorescence signal by either filter sets was also not observed. Fluorescence photomicrography was performed using a Zeiss Axioscope fluorescence microscope with a 40× Neoplan oil objective, N.A. 1.4; Kodak 400ASA Panatomic-x film was exposed for 2 to 8 seconds and processed as 800ASA.

Light microscopic immunoreactivity was semiquantitatively scored. For TF, E-sel, and fibrin, 0 = no reactivity, 1+ = <1 vessel positive/40× field, 2+ = 1 to 5 vessels positive/40× field, and 3+ = >5 vessels positive/40× field. For TM, 0 = no reactivity, 1+ = weak reactivity, 2+ = moderate reactivity, and 3+ = strong reactivity.

Endothelial Cell Culture

Endothelial cells were isolated and propagated from human heart valves as described.[17] Cells were cultured on gelatin-coated tissue culture flasks in medium 199 supplemented with 10% fetal bovine serum, heparin, and ECGS, at 37 C with an atmosphere of 5% CO2. Confluent cell monolayers at passage 4 (1 to 3 split/passage) grown on gelatin-coated glass coverslips were washed with medium 199 and incubated for 6 hours with varying concentrations of LPS in medium 199 plus 10% fetal bovine serum. After washing, coverslips were methanol-fixed and air-dried, then processed for immunohistochemistry as described above for tissue

sections. The percentage of cells reactive for TF or E-sel was determined from counting at least 600 cells in randomly selected fields. Cell monolayers from the same passage grown in 24-well tissue culture trays were similarly stimulated with LPS in parallel, then washed, scraped into assay buffer, and TF activity determined using a clotting time assay as described.[17]

Results

TF and E-sel Expression by LPS-Stimulated Endothelial Cells in Culture

To examine the sensitivity of immunohistochemistry for detecting TF and E-sel, human endothelial cells were grown to confluence on gelatin-coated glass coverslips, incubated with varying concentrations of LPS (0.001 to 100 ng/ml) for 6 hours, then examined immunohistochemically for TF and E-sel, using the same procedures as for frozen tissue sections; simultaneously prepared cell monolayers were assayed for procoagulant activity (Figure 1). Procoagulant activity of lysed cells was concordant with immunoreactivity for TF rather than for E-sel (quantitated as fraction of cells reactive) in the LPS dose-response determinations. In two experiments, the sensitivity of immunocytochemistry was at procoagulant activity (PCA) levels five- to sevenfold above the lower limit of the PCA assay (approximately 5 ng of purified human TF standard per 10^5 cells). Also, to exclude the possibility that *in vivo* TF complexed with factors VIIa, Xa, and tissue factor pathway inhibitor might not be accessible to the anti-TF antibodies, duplicate coverslips of 6 hour–stimulated cells were incubated with recalcified afibrinogenemic plasma (George King Biomedical, Inc., Overland Park, KS) for 15 minutes at 37 C before processing to allow formation of the quarternary complex. No decrease in the frequency or intensity of reactivity was observed in these as compared with the above processed monolayers. There was significant heterogeneity among cells for the presence and extent of immunoreactivity for both TF and E-sel. In dose-response determinations, detection of TF immunoreactivity required approximately 100-fold higher concentrations of LPS than for E-sel immunoreactivity. At the maximal LPS concentration tested, 99% of cells were reactive for E-sel, whereas only approximately 50% expressed detectable TF.

Thus, in this cell culture setting, immunohistochemistry was capable of detecting as little as 5% of the maximum endothelial cell TF response. The greater detectability of E-sel at all LPS concentrations tested suggests a greater concentration of E-sel in cells relative to TF at any given dose. This is supported by other studies we have performed showing that the rate of E-sel messenger RNA transcription is much greater than that of TF in LPS-stimulated endothelial cells (data not presented). It is also consistent with the threshold dose of LPS necessary for initiating synthesis being significantly lower for E-sel than TF.

Biochemical and Physiological Manifestations of Septic Shock

All animals receiving the infusion of live *E. coli* developed shock and disseminated intravascular coagulation. Selected parameters for the five animals sacrificed at 6 to 24 hours are shown in Table 2. Mean systemic arterial pressure fell by more than 50% to approximately 50 mmHg. Platelet counts fell by 60%, whereas fibrinogen concentrations fell to less than 1% baseline values. Fibrin degradation products increased from 10 to 160 ug/dl. Although not determined in blood samples from these animals, previous studies in comparable animals have demonstrated that tumor necrosis factor is consistently detectable in blood from T+1 to T+3 hours, peaking at T+2 hours with concentrations of approximately 100 ng/ml serum.[18]

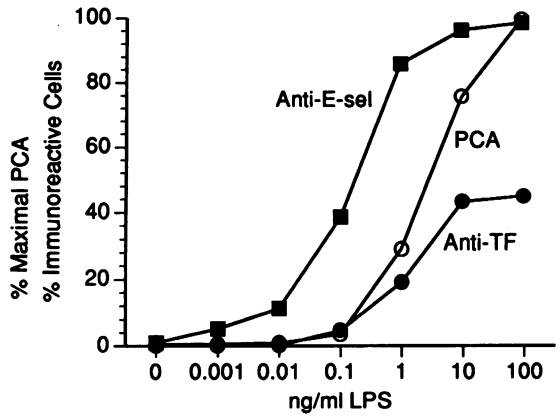

Figure 1. *TF and E-sel immunoreactivity and (PCA) in cultured human endothelial cells exposed to varying concentrations of LPS. Cell monolayers on coverslips were exposed to LPS for 6 hours then processed for immunohistochemistry or PCA. Results for immunoreactivity are expressed as fraction of cells with definite reactivity (600 cells counted per coverslip). PCA was determined by a one-stage clotting assay for duplicate monolayers and results expressed as percent of PCA of cells exposed to 100 ng/ml LPS, in this experiment being equal to 1300 ng of purified TF standard per 10^5 cells (vs 0.5 ng/10^5 cells for unstimulated monolayers). Data shown is for one of two experiments, the second having comparable results.*

Table 2. *Cardiovascular (MSAP), Inflammatory (WBC), Coagulant (Platelets, Fibrinogen, FDP) and Cell Injury (SGPT) Responses to Infusion of LD_{100} E. coli**

	T+0′	T+60′	T+120′	T+180′	T+240′	T+360′
MSAP (mmHg)	116 ± 2	102 ± 4	56 ± 6	62 ± 8	52 ± 4	52 ± 4
WBC ($\times 10^3/mm^3$)	7.3 ± 0.7	3.7 ± 1.0	1.7 ± 0.2	2.3 ± 0.4	2.2 ± 0.4	2.1 ± 0.3
Fibrinogen (% baseline)	100	92 ± 1	88 ± 8	53 ± 15	32 ± 10	<1
FDP (μg/dl)	10					160 ± 22
SGPT (U/dl)	55 ± 18					104 ± 37

* Animals #3, 5, 6, 8 and 10 (n = 5).

Overview of Immunohistochemical Changes in the Microvascular Response to Septic Shock

Septic shock affected the microvasculature of organs throughout the body, as evidenced by the presence of fibrin thrombi and expression of E-sel (Table 3). E-sel was undetectable in all control tissues and present in at least some small vessels in all tissues at T+6 and T+10 hours, indicating widespread endothelial activation. Despite the sensitivity of the immunohistochemical technique used, TF was unequivocally detected only in the spleen and possibly expressed in renal glomerular and lung alveolar capillaries, as detailed below. TM immunoreactivity was present in microvascular endothelium of all control animals and remained essentially unchanged in septic animals (up to T+24 hours).

Comparison of sites of fibrin deposition, E-sel, TM, and TF expression (Table 3) do not demonstrate any uniformly consistent associations. However, using E-sel or TF expression as an indicator of endothelial activation, only in the alveolar septal vessels were thrombi present in the absence of detectable endothelial activation.

Demonstration and Localization of Endothelial Cell (TM, TF, and E-sel) Response to E. coli in the Spleen, Liver, and Skin

General Observations—Spleen, Liver, and Skin

In control animals and in biopsies obtained at T-0, TM was detectable with 2 to 3+ reactivity in the endothelium of all vessels in these organs, whereas TF and E-sel were undetectable. TF however was detectable extravascularly in epidermal keratinocytes, adventitial fibroblasts of medium-sized arteries and veins, and cells of the splenic trabeculae and capsule, as observed in man.[16] Immunohistochemical examination of sequential biopsies of spleen, liver, and skin of animals #6 and #8 revealed microvascular thrombi and endothelial activation as indicated by induction of E-sel in vessels of all three organs within 2 to 6 hours of initiation of bacteremia. However, TF became detectable only in the spleen, and TM remained constant except for a modest apparent decrease in the liver of 1 of the animals (Table 3, Figure 2). Results were similar for the additional animals examined only at

Table 3. *Fibrin, E-sel, TF, and TM Immunoreactivity in the Microvasculature of Organs of Animals #6, #8, and #3 at T+6 Hours following E. coli infusion*

	FIBRIN	E-sel	TF	TM
Spleen, marginal zone	+++	0	+++	+++
Spleen, sinusoids	+++	+++	0	+++
Liver, sinusoids	+++	+ to ++	0	+ to ++
Liver, central veins	+ to ++	++ to +++	0	++ to +++
Kidney, glomeruli	+++	+++	?	0
Kidney, peritubular	+++	+++	0	+++
Adrenal cortex	+++	+++	0	+ to ++
Adrenal medulla	+++	++ to +++	0	++
Myocardium	+	+ to ++	0	+++
Lung, alveoli	0 to +++	0	?	+++
Cerebral cortex	+ to ++	+++	0	0
Skin, dermis	0 to ++	++	0	+++
Aorta	0	0	0	+++

In tissues of control animals, fibrin reactivity was 0 to +, E-sel reactivity 0, TF reactivity 0 except in glomeruli and lung as discussed in text, and TM reactivity similar to data shown for T+6 hours.

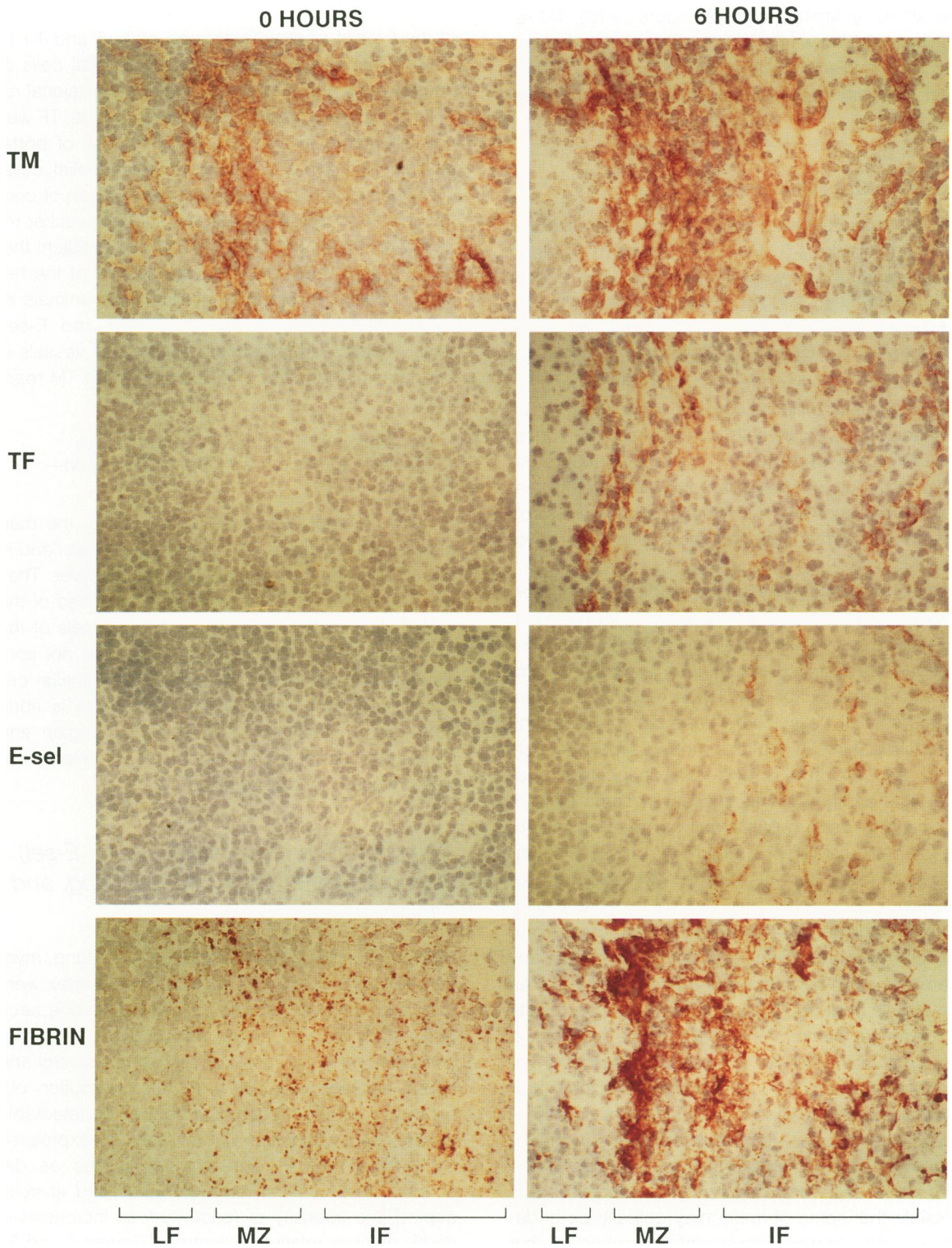

Figure 2. *Immunohistochemical localization of TM, TF, E-sel, and fibrin in the spleen of animal #6 at T-0 and T+6 hours. TM, TF, and fibrin are present in both the marginal (MZ) and interfollicular (IF) zones, whereas E-sel is present only in sinusoidal endothelium of the interfollicular zone; (LF indicates lymphoid follicle). Magnification: ×200.*

sacrifice at T+6 (#3) and T+10 hours (#5). For the one animal examined at T+24 hours (#10), TM reactivity in spleen was diminished (1 to 2+), whereas reactivity in liver and skin was unchanged. TF and E-sel reactivity had diminished to low or undetectable levels.

Specific Observations—Spleen

TM immunoreactivity was strongly expressed at all time intervals (T-0, T+2, T+6 hours) by cells in the marginal zones, whereas reactivity was present but variable (1 to 3+) for sinusoidal cells in the interfollicular zones (Figure 2). TF expression in response to *E. coli* reached a maximum at T+6 hours in the marginal zone regions and less prominently in cells of the interfollicular regions. In contrast, E-sel was not observed in cells of the marginal zones; however, there were numerous reactive cells of sinusoids in the interfollicular and subcapsular regions. The endothelium of small and medium-sized veins and of small arterioles were also positive for E-sel.

Dual immunofluorescence studies were performed to determine whether some of the TF reactive cells in the spleen of animals at T+6 hours were vascular endothelial cells (Figure 3). Both TM and von Willebrand factor (vWF) were used as markers for endothelial cells. Three important observations were made. First, in sections probed simultaneously for TF and TM, and TF and vWF, co-localization of fluorescence signals was present in some cells of the marginal zone but this was not true for cells of the interfollicular regions (Figure 3, A to D). Simultaneous probing for E-sel and TM showed co-localization of signals from sinusoidal endothelium as expected (data not shown). Second, dual immunofluorescence for E-sel and TF did not reveal any examples of co-expression of these proteins by the same cells (Figure 3, G and H). Thus these results suggest that some of the cells in the spleen expressing TF are endothelial cells but that this is limited to the marginal zone of lymphoid follicles. Third, simultaneous probing for TF and CD68 showed co-localization of signals from macrophages in the interfollicular regions (Figure 3, E and F). Most of the TF-positive cells, however, remain unidentified. It is possible that some of these may actually be endothelial cells and/or monocyte/macrophages that have undergone simultaneous down-regulation of the markers used to identify the cell types. Finally, it should be noted that there was no co-localization of anti-CD68 with anti-TM or anti-E-sel to cells in any area (data not shown).

Specific Observations—Liver and Skin

In the liver of animals examined at T+6 and T+10 hours E-sel was expressed by endothelial cells of central veins and portal vessels, with occasional reactivity in sinusoidal cells near central veins. TF was not detectable except in adventitial cells of portal vessels and some biliary ductular epithelial cells. TM was strongly expressed by endothelium of central veins and portal vessels but showed weaker reactivity in sinusoidal lining cells, with a gradient that became less intense in the outer zones of the hepatic lobules. In samples of skin from animals at these times, scattered fibrin thrombi and E-sel-positive cells were present in some small vessels in the dermis. These had consistently strong TM reactivity.

Correlation of Endothelial Cell Response (TF, E-sel Expression) with Fibrin Deposition—Spleen and Liver

Fibrin thrombi were most prominent in the marginal zones of lymphoid follicles in the spleen and in sinusoids near the central veins of the liver. They were also present in the interfollicular zones of the spleen. None were present in portal vessels of the liver. Thus, localization of fibrin thrombi is not consistently correlated with detectable endothelial cell activation. For instance, in the liver there is fibrin deposition in the central vein/sinusoid region and none in the portal region, whereas E-sel expression was observed in both regions.

Demonstration of Cellular (TM, TF, E-sel) Response to E. coli in Kidney, Lung, and Other Tissues

Sections of kidney, adrenal gland, aorta, lung, myocardium, skeletal muscle, and cerebral cortex were examined in three animals at T+6 hours, one each at T+10 and T+24 hours, and in two controls for comparison. TF expression in organs of control and septic animals was similar to the distribution observed in normal human tissues, i.e., undetectable in vascular endothelium but differentially expressed by various cells in extravascular tissues as described. At two sites, renal glomeruli and alveolar septae, the intensity of reactivity was increased in septic animals relative to controls (Figures 4 and 5). In renal glomeruli, where fibrin deposition in capillary loops was prominent, there was diffuse TF immunoreactivity (Figure 4) and concomitant expression of E-sel by glomerular capillary endothelium. TM however was weak to absent in the glomeruli of

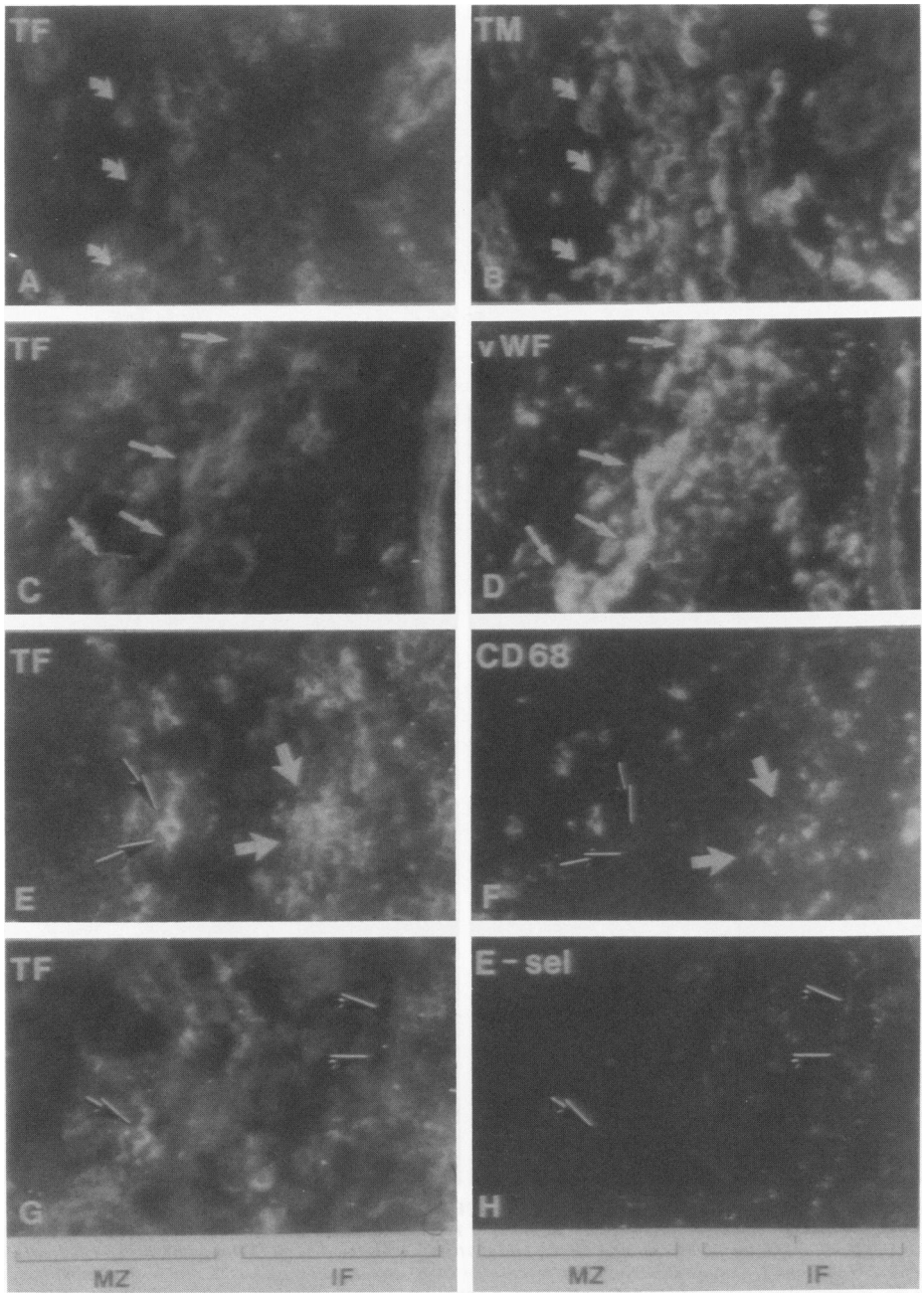

Figure 3. *Dual immunofluorescence for TF and TM* (A *and* B), *TF and vWF* (C *and* D), *TF and CD-68, a macrophage marker* (E *and* F), *and TF and E-sel* (G *and* H) *in splenic tissue of animal #6 at T+6 hours. TF co-localizes with marginal zone vascular structures that express TM and vWF (solid arrows,* A *to* D) *but not the macrophage marker CD-68 (shadowed arrows,* E *and* F), *demonstrating TF expression by endothelial cells. (Solid arrows in* E *and* F *point to focus of co-localization of TF and CD 68 signals in the interfollicular zone). Sinusoidal endothelium in the interfollicular zone that expresses E-sel* (G *and* H, *arrowheads) does not express detectable TF, whereas TF-positive structure in marginal zone* (G *and* H, *arrow) does not express E-sel. Anatomical orientation is similar to Figure 1, with marginal zone on the left half of each photomicrograph, the interfollicular zone on the right. Magnification:* A *to* D, ×*750;* E *to* H, ×*480.*

both control and septic animals, except for endothelial cells of the apparent afferent arteriole entering the glomerular tuft. To identify the principal cells expressing TF, dual immunofluorescence on 4-μ-thick sections was performed for TF and CD10 (common acute lymphoblastic leukemia associated antigen or CALLA), TF and vimentin, and TF and E-sel. In human renal glomeruli, CD10 is expressed only by glomerular epithelial cells[19] whereas vimentin is expressed by epithelial, mesangial, and endothelial cells.[20] CD10-positive structures were nearly always TF-positive (Figure 4, C and D), indicating that epithelial cells accounted for over 90% of TF positivity in glomeruli. Some limited areas immu-

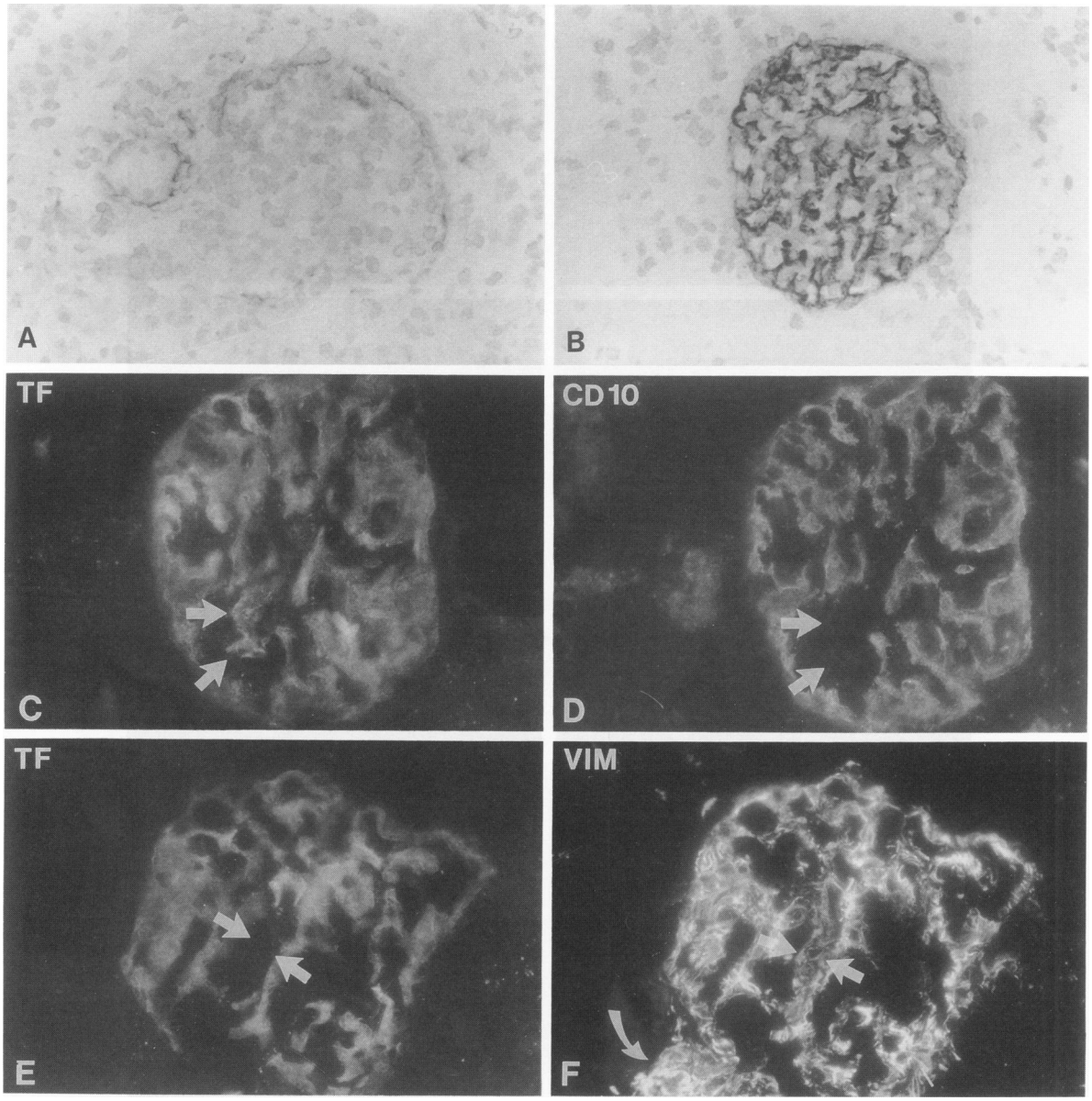

Figure 4. *Induction of TF immunoreactivity in renal glomerulus* (A *and* B *and co-localization of TF expression with glomerular epithelial cells in septic animals* (C *to* F). A *and* B*: Immunohistochemical localization of TF in renal glomerulus of control* (A) *and septic* (B*; animal #3 at T+6 hours) animals.* C *and* D*: dual immunofluorescence for TF* (C) *and CD10* (D)*, showing that all CD10- (epithelial specific marker) positive structures are reactive for TF (also, one TF-positive structure is not reactive for CD10, indicated by arrows).* E *and* F*: dual immunofluorescence for TF* (E) *and vimentin* (F)*, showing that most but not all vimentin-positive areas are TF-positive; straight arrows indicate an apparent mesangial area that is vimentin-positive TF-negative; the curved arrow in* (F) *points to an afferent arteriole adjacent to the glomerulus. Magnification:* A *and* B*, ×200;* C *to* F*, ×480.*

noreactive for TF were CD10-negative as demonstrated in Figure 4, C and D. Because epithelial cells also express vimentin, TF also co-localized with it in most areas. Consistently present however were centrally located vimentin-positive/TF-negative areas that were considered to be mesangial because they did not have endothelial morphology (Figure 4, E and F). Therefore TF reactivity in glomeruli seemed to be due mainly to its expression by epithelial rather than mesangial cells. Dual immunofluorescence for TF and E-sel showed limited areas of co-localization, but the close proximity of endothelial cells with the strongly TF-positive epithelial cells precluded a definitive assessment of its potential expression by capillary endothelium using this technique.

In the lung, a subpopulation of cells of the alveolar septae showed strong TF reactivity relative to controls. These were identifiable by dual immunofluorescence for TF and cytokeratin as epithelial cells

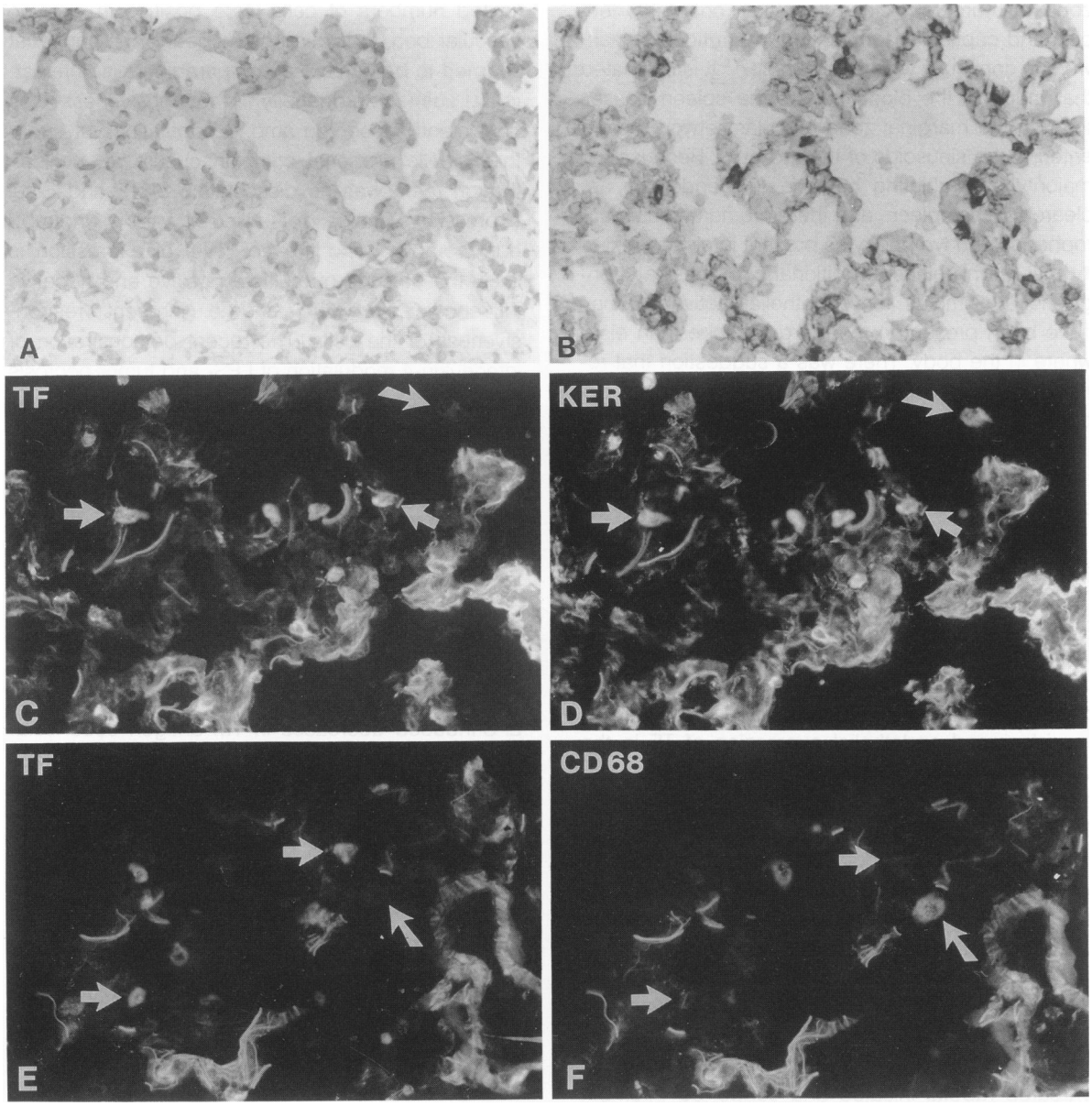

Figure 5. *Induction of TF immunoreactivity in lung alveolar septae* (**A** *and* **B**) *and co-localization of TF expression with alveolar epithelial cells* (**C** *and* **D**) *but not alveolar macrophages* (**E** *and* **F**) *in a septic animal.* **A** *and* **B**: *Immunohistochemical localization of TF in alveolar septae of control* (**A**) *and septic* (**B**: *animal # 5 at T+ 10 hours*) *animals.* **C** *and* **D**: *dual immunofluorescence for TF* (**C**) *and the epithelial cell marker cytokeratin* (**D**). **E** *and* **F**: *dual immunofluorescence for TF* (**E**) *and the macrophage marker CD68* (**F**) *in tissue from animal # 5 at T+ 10 hours. Cells that are strongly reactive for TF (straight arrows) also express cytokeratin but not CD-68, indicating they are of epithelial origin. The curved arrows point to positions of cells reactive for cytokeratin* (**C** *and* **D**) *or CD 68* (**E** *and* **F**) *but not TF. Magnification:* **A** *and* **B**, *×200;* **C** *to* **F**, *×480.*

(Figure 5, C and D). Interestingly, TF expression by alveolar cells identified as macrophages with anti-CD68 was weak to absent in septic animals (Figure 5, E and F), consistent with observations for monocyte-derived cells in other organs apart from the spleen. Although fibrin thrombi were present in some alveolar capillaries, immunoreactive fibrin was not prominent in alveolar spaces. E-sel was not detected in alveolar capillary endothelium but was present in endothelium of venules and peribronchial vessels.

Discussion

Induced endothelial expression of TF was documented in the microvasculature of the spleen by dual immunofluorescent co-localization of TF with both TM and vWF, endothelial cell–specific markers. The time course of expression paralleled that observed in cultured endothelial cells, beginning at 2 hours, peaking at 6 to 10 hours, and decreasing to low levels at 24 hours. This occurred in the marginal zones surrounding lymphoid follicles. The marginal

zone has numerous circumferentially oriented arterioles and capillaries terminating in a reticular meshwork in continuity with the red pulp.[21] It is estimated that much of the blood flow to the spleen passes through the marginal zone reticular network before entering the sinusoids of the red pulp. Because the majority of circulating bacteria and endotoxin are cleared in the spleen, and there are numerous macrophages and lymphocytes present in close proximity that might release additional cytokines, a stronger local stimulus for endothelial activation may have been present in the marginal zone than elsewhere in the body. There was a suggestion that renal glomerular capillary endothelium may also express TF, although more precise localization by ultrastructural analysis will be required to unequivocally distinguish endothelial from epithelial cell TF expression.

Despite expression of E-sel in all other organs examined, apparent endothelial expression of TF was not observed except in the spleen. Because TF could be detected immunohistochemically in LPS-stimulated cultured human endothelial cells that expressed as little as 5% of the maximum inducible procoagulant activity, one can conclude that if there is widespread endothelial expression of TF in the baboon model of septic shock, the level of expression must be far less than that which occurs *in vitro*. There is therefore a previously unappreciated physiological control of endothelial cell TF expression *in vivo*, modulating the multiple strong stimuli present in lethal *E. coli* septic shock. Concomitantly, there could be relatively low yet significant levels of TF induction occurring because the degree of TF expression that might be pathogenetically significant *in vivo* is not known. Nawroth and Stern have reported that aortas freshly removed from interleukin-1-treated rabbits initiated and supported thrombin generation on the luminal surface when incubated with factor VIIa, factor X, and prothrombin, indicating that TF was present on the surface of luminal endothelial cells.[22] Previous physiological studies with the baboon model unequivocally indicate that activation of coagulation is principally via the TF pathway *in vivo*.[23] Circulating monocytes also typically express TF when activated and are very likely a potent source of intravascular TF in septic shock.[24] However, the relative contribution of endothelium and monocytes cannot be assessed from this study.

Our observations also suggest that endothelial activation *in vivo* is a heterogeneous phenomenon. At least quantitatively if not qualitatively, expression of TF was distinct from E-sel, indeed it seemed mutually exclusive. Also, for both TF and E-sel, only limited numbers of cells were positive in a given vascular bed, indicating heterogeneity among what seemed to be otherwise comparable cells. Interestingly, such heterogeneity in responsiveness was consistently observed among populations of endothelial cells of clonal origin in culture as well, although there was no dissociation of TF and E-sel expression. The cellular milieu *in vivo* is undoubtedly complex, and the net level of expression is likely the balance of multiple positive and negative influences, as well as the inherent responsiveness of the cells. The recent demonstration of interleukin-4 modulation of LPS- and tumor necrosis factor-induced TM down-regulation is one such example.[25] Elucidation of the transcriptional regulation of these genes will also help in understanding and perhaps predicting *in vivo* behavior.[26]

Down-regulation of TM could not be demonstrated with certainty in any vascular bed, suggesting that TM regulation *in vivo* may differ from that in cultured cells. However, this interpretation must be made with caution as the results do not exclude the possibility that functionally significant changes in TM did occur *in vivo*. In cultured endothelial cells, LPS-induced decreases in TM antigen occur relatively slowly, and even at the 24-hour time point may be of limited magnitude and therefore difficult to detect by a semiquantitative technique.[7,8] More importantly, in the process of down-regulation, TM may be internalized and degraded intracellularly, and light microscopic immunocytochemistry on tissue sections would not distinguish functional (i.e., surface-expressed) from internalized immunoreactive protein.[27] Microvascular thrombi clearly show a predeliction for certain organs, which is most likely explained by variations in the local microvascular response that we have not identified. Future studies should pursue ultrastructural immunolocalization of TM, and evaluation of other endothelial cell hemostatic properties such as plasminogen activator inhibitor type 1.

An interesting finding was the induction or up-regulation of TF expression by epithelial cells of renal glomeruli and of lung alveoli, compared with the, at most, limited induction in monocyte-derived cells in these organs. The kidney and lung are relatively sensitive to injury in septic shock, and it is possible that enhanced TF expression plays a role in the pathophysiology of injury. Much of the investigation of induced procoagulant activity in lung injury has focused on alveolar macrophages.[28] Our findings support the suggestions of Gross et al that attention should be directed to the epithelial cells as well.[29]

In summary, we have documented that endothelial cells can express TF *in vivo* in a relevant pathological setting, but at the same time found that this was restricted to a few vascular beds at most rather than being the widespread phenomenon anticipated. Where present, TF expression was dissociated from that of E-sel, indicating that the activation response of endothelium *in vivo* is more heterogenous than *in vitro*. Further studies are needed to elucidate the role of endothelium in microvascular thrombosis and to determine the local factors that influence the net hemostatic function in the microvasculature *in vivo*. Because thrombin is a potent agonist for a variety of cellular responses,[30,31] the significance of potential alterations in endothelial hemostatic function may be quite pleiotropic.

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